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Red wine and oenological extracts display antimicrobial effects in an oral bacteria biofilm model

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Abstract: The antimicrobial effects of red wine and its inherent components on oral microbiota were studied by using a 5-species biofilm model of the supragingival plaque that includes *Actinomyces oris*, *Fusobacterium nucleatum*, *Streptococcus oralis*, *Streptococcus mutans* and *Veillonella dispar*. Microbiological analysis (CFU counting and confocal laser scanning microscopy) of the biofilms after the application of red wine, dealcoholized red wine, and red wine extract solutions spiked or not with grape seed and inactive dry yeast extracts showed that the solutions spiked with seed extract were effective against *F. nucleatum*, *S. oralis* and *A. oris*. Also, red wine and dealcoholized wine had an antimicrobial effect against *F. nucleatum* and *S. oralis*. Additional experiments showed almost complete and early degradation of flavan-3-ol precursors [(+)-catechin and procyanidin B2] when incubating biofilms with the red wine extract. To our knowledge, this is the first study of antimicrobial properties of wine in an oral biofilm model.

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1 **Red wine and polyphenols display antimicrobial effects in an oral bacteria biofilm model**

2

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16 *Running head: Oral biofilm antimicrobial effects of red wine*

17 *Keywords: wine, polyphenols, oral bacteria biofilm, antimicrobials*

18

19 **Abstract**

20

21 The antimicrobial effects of red wine and its inherent components on oral microbiota have
22 been studied by using a 5-species biofilm model of the supragingival plaque that includes
23 *Actinomyces oris*, *Fusobacterium nucleatum*, *Streptococcus oralis*, *Streptococcus mutans* and
24 *Veillonella dispar*.

25 Microbiological analysis (CFU counting and confocal laser scanning microscopy) of the
26 biofilms after the application of different test solutions (red wine, dealcoholized wine, red wine
27 extract, grape seed extract and inactive dry yeast extracts) showed that the grape seed extract
28 solution was the most effective, exhibiting high activity against *F. nucleatum*, *S. oralis* and *A.*
29 *oris*. Additionally, both red wine and dealcoholized red wine had an antimicrobial effect
30 against *F. nucleatum* and *S. oralis*.

31 Additional experiments were carried out to determine any possible phenolic metabolism during
32 formation of the bacterial biofilm. Flavan-3-ol precursors such as (+)-catechin and procyanidin
33 B2 suffered an almost complete and early degradation when incubating biofilms with the red
34 wine extract but no degradation was observed after incubation with the grape seed extract,
35 probably due both to its higher concentration and antimicrobial effects. To our knowledge, this
36 is the first study of antimicrobial properties of wine in an oral biofilm model.

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46 **Introduction**

47 The oral cavity is an enormously complex habitat with several hundred commensal microbial
48 species colonizing it, and furthermore it is unique in the human body in possessing non-
49 shedding surfaces, the teeth, allowing microorganisms to adhere to the surface of teeth for long
50 periods of time, embedded in a self-produced matrix of extracellular polymeric substances
51 (Abee *et al.*, 2011), and thus leading to extensive biofilm formation, dental plaque (Marsh,
52 2003), which is more resistant than planktonic cells to mechanical stress or antibiotic treatment
53 (Roberts *et al.*, 2010). The microorganisms of dental plaque live with one another in a
54 commensal or mutualistic symbiotic relationship, allowing a mixture of aerobic and anaerobic
55 bacteria to live in the same environment. Some of these oral bacteria such as streptococci or
56 lactobacilli are able to produce high levels of organic acids following fermentation of dietary
57 sugars. Acids released from dental plaque lead to demineralization of the tooth surface and
58 consequently to dental caries, periodontal disease or tooth loss (Hardie, 1992), which are the
59 most prevalent oral diseases in humans, affecting up to 60–90% of the world population (da
60 Silva *et al.*, 2013).

61 Even using mechanical removal, dental biofilms cannot be eliminated completely.
62 Antimicrobial agents are complementarily used to control dental plaque (Furiga *et al.*, 2008;
63 Marsh *et al.*, 2010; Kamonpatana *et al.*, 2012). Until now, several substances have been tested
64 for the control of oral biofilms, including essential oils, amine fluoride, triclosan, etc., but one
65 of the most widely used and effective antibiofilm agents is chlorhexidine (Corbin *et al.*, 2011).
66 However, chlorhexidine has been associated with some secondary effects, namely the
67 reduction of human taste perception and the pigmentation of oral tissues, which limits its
68 application. Therefore, the search for new antimicrobials has arisen, and natural products are
69 preferable due to the lack of secondary effects and therefore, the potential for long-term usage
70 in the oral cavity.

71 The inherent matrix of the biofilm, such as extracellular polymeric substances that reduce
72 penetration of antimicrobial agents and the presence of persister cells surviving at low
73 metabolic rates, contributes to the widely described phenomenon of reduced sensitivity to
74 antimicrobial agents (Hoyle & Costerton, 1991). Because of this, biofilm models including
75 bacteria and fungi from different species have proven both useful and reliable and reliability in

76 predicting *in vivo* efficacy of antimicrobials. In this sense, most experimental models for short-
77 term studies involve a solid surface for the adhesion of bacteria (Guggenheim *et al.*, 2004).

78 Although there is substantial literature reporting the antimicrobial properties of phenolic
79 compounds or polyphenols against bacteria isolates (Jayaprakasha *et al.*, 2003; Ozkan *et al.*,
80 2004; Cueva *et al.*, 2010), information about their effect on oral pathogens is still scarce
81 (Requena *et al.*, 2010). Studies carried out with tea and cranberry polyphenols have shown an
82 inhibitory effect on biofilm formation by oral pathogens such as *Streptococcus mutans*,
83 *Streptococcus oralis*, *Streptococcus sobrinus* and *Porphyromonas gingivalis* (Bodet *et al.*,
84 2008). Grapes and wines are good dietary sources of polyphenolic compounds, including
85 hydroxybenzoic and hydroxycinnamic acids, phenolic alcohols, flavan-3-ol monomers,
86 oligomeric and polymeric procyanidins, flavonols, stilbenes and anthocyanins (only present in
87 red varieties) (Monagas *et al.*, 2003). Recently, it has been found that wine and grape phenolic
88 extracts, as well as pomace phenolic extracts, were able to inhibit the growth of different
89 *Streptococcus* spp. strains associated with dental caries (Thimothe *et al.*, 2007; Furiga *et al.*,
90 2009).

91 On the other hand, interactions between wine phenolics and oral microbiota can also include a
92 possible bacterial catabolism of wine phenolics into less complex phenolic metabolite
93 structures as seems to happen with flavonol glycosides (Requena *et al.*, 2010). With regards to
94 anthocyanins, their degradation in human saliva at 37 °C has been described, being structure-
95 dependent, largely mediated by oral microbiota, and partially suppressed after oral rinsing with
96 antibacterial chlorhexidine (Kamonpatana *et al.*, 2012).

97 With the final aim of seeking natural products that could be used in oral hygiene and to
98 ascertain interactions between wine components and oral microbiota, in this study the
99 antimicrobial effects of red wine and dealcoholized red wine were investigated using a biofilm
100 model of the supragingival plaque that integrates five bacteria species commonly associated
101 with oral disease. A wine phenolic extract (Provinols™), especially rich in anthocyanins, was
102 also tested using the same model, and in both the absence and presence of other enological
103 extracts from grape seeds (Vitaflavan®) and yeast (inactive dry yeast, IDY). Additional
104 experiments were carried out to determine any possible phenolic metabolism during the
105 formation of the bacterial biofilm.

106

107 **Materials and methods**

108 *Red wines*

109 The red wine used in this study was a young red wine (var. Pinot Noir, vintage 2010), kindly
110 provided by Bodegas Miguel Torres S.A. (Catalonia, Spain). The wine was elaborated
111 following the winery's own winemaking procedures and was selected because of its relatively
112 high phenolic content: total polyphenols = 1758 mg of gallic acid equivalents/L, total
113 anthocyanins = 447 mg of malvidin-3-glucoside/L, and total catechins = 1612 mg of (+)-
114 catechin/L. Main individual phenolic compounds found in this wine included anthocyanins,
115 flavan-3-ols, flavonols, alcohols, stilbenes and hydroxycinnamic acids (Muñoz-González *et al.*,
116 2013) (Table 1).

117 For the preparation of dealcoholized red wine, ethanol was removed using a rotary evaporator
118 and then distilled water was added until the original volume was reached.

119

120 *Enological extracts*

121 A wine extract, Provinols™, was kindly supplied by Safic-Alcan Especialidades S.A.U.
122 (Barcelona, Spain). A grape seed extract, Vitaflavan® was kindly provided by Dr. Piriou (Les
123 Dérivés Resiniques & Terpéniques S.A., France). The total phenolic content of the extracts was
124 474 mg of gallic acid equivalents/g for Provinols™ and 629 mg of gallic acid equivalents/g for
125 Vitaflavan®. The main phenolic compounds identified in both extracts are reported in Table 1.
126 Also, two inactive dry yeast (IDY) commercial preparations (*Saccharomyces cerevisiae*), IDY
127 1 and IDY 2, rich in mannoproteins, aminoacids and peptides, respectively, were kindly
128 provided by Lallemand S.A. (Blagnac, France) and Agrovin S.A. (Alcázar de San Juan, Ciudad
129 Real, Spain).

130 The wine extract was dissolved in distilled water containing 2.5% DMSO (v/v), at a
131 concentration of 1.6 g/L. The wine extract solution was fortified in grape seed polyphenols by
132 adding 2.5 g of grape seed extract to 100 mL of the wine solution. Also, the wine extract
133 solution was enriched in wine matrix components (mainly polysaccharides and nitrogen

134 compounds) by adding the IDY preparations to the wine extract solution at a final
135 concentration of 0.4 g/L.

136

137 *Bacterial strains and culture (growth) conditions*

138 *Actinomyces oris* OMZ 745, *Fusobacterium nucleatum* OMZ 598, *Streptococcus oralis* OMZ
139 607, *Streptococcus mutans* UA159 (OMZ 918) and *Veillonella dispar* ATCC 17748^T (OMZ
140 493) were obtained from the culture collection of the Institute of Oral Biology, University of
141 Zürich. Prior to the experiment, pre-cultures were prepared by transferring the strains on
142 Columbia Blood Agar plates and incubating them for 96 h at 37 °C under anaerobic conditions.
143 After this time, the strains were transferred from the Columbia Blood Agar plates to broth
144 cultures (1 x 9 ml FUM in Sørensen's buffer + 0.3 % glucose) (OMZ 493: + 1% sodium
145 lactate) and incubated overnight at 37 °C. After incubation, 200 µL of bacteria from each
146 working culture were individually inoculated in 5 mL of fresh FUM media in Sørensen's
147 buffer and incubated at 37 °C anaerobically (7 hours maximum). In order to obtain an
148 inoculum containing cultures in the exponential growth phase of approximately 10⁷ CFU/mL, a
149 microbial suspension with equal volumes and densities of each strain was prepared.

150

151 *Saliva processing*

152 Saliva was collected and processed according to the protocol of Guggenheim *et al.* (2001).
153 Briefly, whole unstimulated saliva was collected from volunteers for 1 h each morning, over
154 several days, at least 1.5 h after eating, drinking, or teeth cleaning. Saliva samples were
155 collected in sterile 50 mL polypropylene tubes, chilled in an ice bath or frozen at -20 °C. After
156 500 mL saliva had been collected, it was pooled and centrifuged (30 min, 4 °C, 27,000 × g);
157 the supernatant was pasteurized (60 °C, 30 min) and re-centrifuged in sterile tubes. The
158 resulting supernatant was stored in sterile 50 mL polypropylene tubes at -80 °C. The
159 efficiency of the process was assessed by plating the processed saliva samples onto CBA agar;
160 after 72 h at 37 °C, no CFUs were observed on the incubated plates. A sterile 1:1 dilution in
161 H₂O+25% physiological NaCl was used for the biofilm formation and throughout the
162 experimentation.

163

164 *In vitro biofilm experiments*

165 Figure 1 shows a sequence chart regarding the biofilm formation prior to assays for
166 determining changes in the microbial population of the biofilm and for assessing phenolic
167 metabolism in the biofilm.

168

169 *Biofilm formation*

170 Biofilms were grown using the slightly modified protocol described by Guggenheim *et al.*
171 (2001) and Thurnheer *et al.* (2006). In brief, the 5-species biofilms were grown in 24-well
172 polystyrene cell-culture plates on hydroxyapatite (HA) discs of 9mm Ø (Clarkson
173 Chromatography Products, South Williamsport, USA) previously preconditioned in 800 µL of
174 whole unstimulated pooled saliva (as described in the previous section) during 4 h at room
175 temperature, with shaking (95 rpm) in order to promote pellicle formation. To initiate the
176 biofilm formation, the discs were covered for 45 minutes with 1.6 mL of a mixture comprising
177 30% saliva, 70% modified fluid universal medium (mFUM) and 200 µL of the bacterial
178 inoculum described above. mFUM corresponds to a well-established tryptone yeast-based
179 broth medium designated as FUM (Gmür & Guggenheim, 1983) and modified by
180 supplementing 67 mM Sørensen's buffer (final pH 7.2). The carbohydrate concentration in
181 mFUM was 0.3% (w/v) and consisted of glucose for the first 16 h and from then on of a 1:1
182 (w/w) mixture of glucose and sucrose.

183 After this first incubation, discs were subjected to three consecutive 1 min dip-washes in 2 ml
184 0.9% NaCl to remove growth medium and free-floating cells but not microorganisms adhering
185 firmly to the HA discs. Then, they were incubated anaerobically for 16.5 h at 37 °C in
186 preconditioned and processed saliva to form the biofilm (Figure 1).

187

188 *Assay for determining changes in the microbial population of the biofilm*

189 Once the biofilm was formed, discs were maintained in a 24-well plate with preconditioned
190 and processed saliva in anaerobic conditions for 7 days. Twice a day, and with 7 hours of
191 difference in between, discs were “fed” by immersing them into a preconditioned fresh growth
192 medium (30% saliva, 70% mFUM (v/v) containing 0.15% glucose and 0.15 % sucrose) for 45

193 minutes, at 37 °C, under anaerobic conditions, After each “feeding”, discs were dipped in the
194 different test solutions (1 mL) for 2 minutes and while being gently shaken by hand. After this
195 time, the discs were dipped once in the preconditioned-processed saliva in order to clean any
196 remains of the test solutions. Immediately after, discs were returned to the “old” 24-well plate
197 with preconditioned and processed saliva and incubated anaerobically until the next “feeding”
198 (Figure 1). After 7 days, biofilms were either stained for confocal laser scanning microscopy
199 (see below) or harvested, at room temperature, in 1 mL of 0.9% NaCl by scratching with a
200 special odontological instrument. Cell viability was tested using a Live/Dead BacLight
201 Viability Kit (Molecular Probes Inc.) The total CFU, streptococci and all taxa were assessed by
202 anaerobic culture (37 °C) using selective (Mitis Salivarius for *Streptococcus oralis* and
203 *Streptococcus mutans*; Fastidious Anaerobe Agar for *Fusobacterium nucleatum*) and non-
204 selective media (Columbia Blood Agar for *Actinomyces oris*, *Veillonella dispar* and total CFU)
205 and colonies were counted.

206 Distilled water was used as the negative antimicrobial control, and 0.2% chlorhexidine-
207 gluconate solution (Sigma-Aldrich, Steinheim, Germany) in water was the positive
208 antimicrobial control. In order to discard a possible antimicrobial effect of the alcohol 12%
209 ethanol in water was also tested. For both, test solutions and controls, experiments were carried
210 out in triplicate.

211

212 *Assay for assessing phenolic metabolism in the biofilm*

213 After initiating biofilm formation as described above, the 70:30 saliva:mFUM media was
214 enriched with the wine extract (1.6 g/L) in the absence of the presence of grape seed extract
215 (10 g/L) and added into the wells containing the discs (Figure 1). Then, plates were incubated
216 at 37 °C under anaerobic conditions and aliquots of enriched media were taken at 0, 2, 4, 6, 8
217 and 24 hours.

218

219 *Analysis of wine compounds and bacterial/microbial metabolites*

220 Phenolic compounds were analyzed using an UPLC-ESI-MS/MS following a previously
221 reported method (Muñoz-González *et al.*, 2013). The liquid chromatographic system was a
222 Waters Acquity UPLC (Milford, MA) equipped with a binary pump, an autosampler

223 thermostatted at 10 °C, and a heated column compartment (40 °C). The column employed was
224 a BEH-C18, 2.1 x 100 mm and 1.7 µm particle size from Waters (Milford, MA). The mobile
225 phases were 2% acetic acid in water (A) and 2% acetic acid in acetonitrile (B). The gradient
226 program was as follows: 0 min, 0.1% B; 1.5 min, 0.1% B; 11.17 min, 16.3% B; 11.5 min,
227 18.4% B; 14 min, 18.4% B; 14.1 min, 99.9% B; 15.5 min, 99.9% B; 15.6 min, 0.1% B.
228 Equilibrium time was 2.4 min resulting in a total runtime of 18 min. The flow rate was set
229 constant at 0.5 mL/min and injection volume was 2 µL.

230 The LC effluent was pumped to an Acquity TQD tandem quadrupole mass spectrometer
231 equipped with a Z-spray electrospray ionization (ESI) source operated in negative polarity
232 mode. The ESI parameters were set as follows: capillary voltage, 3 kV; source temperature,
233 130 °C; desolvation temperature, 400 °C; desolvation gas (N₂) flow rate, 750 L/h; cone gas
234 (N₂) flow rate, 60 L/h. The ESI was operated in negative ionization mode. For quantification
235 purposes, data were collected in the multiple reaction monitoring (MRM) mode, tracking the
236 transition of parent and product ions specific to each compound. The MS/MS parameters (cone
237 voltage, collision energy and MRM transition) of the 60 phenolic compounds targeted in the
238 present study (mandelic acids, benzoic acids, phenols, hippuric acids, phenylacetic acids,
239 phenylpropionic acids, cinnamic acids, 4-hydroxyvaleric acids and valerolactones) were
240 previously reported (Jiménez-Girón *et al.*, 2013). Data acquisition and processing was realized
241 with MassLynx 4.1 software.

242

243 *Staining of biofilms and confocal laser scanning microscopy (CLSM)*

244 For CLSM, treated as well as untreated biofilms were stained using the LIVE/DEAD BacLight
245 bacterial viability assay (Invitrogen, Zug, Switzerland) according to the instructions of the
246 manufacturer. After 20 min staining, excess dye was gently aspirated from the discs without
247 touching the biofilms. They were embedded upside-down in 20 µl of Mowiol (Thurnheer *et al.*,
248 2003) and stored at room temperature in the dark for at least 6 h prior to microscopic
249 examination.

250 Stained biofilms were examined by CLSM at randomly selected positions using a Leica TCS
251 SP5 (Leica Microsystems, Heidelberg GmbH, Germany) with a x20/0.8 numerical aperture
252 (NA) and x63/1.4 NA oil immersion objective lens in conjunction with 488-nm laser excitation

253 and 530-nm emission filters for Syto 9 (live stain), and 561-nm laser excitation and 640-nm
254 emission filters for propidium iodide (dead stain). Image acquisition was done in 8-line
255 average mode and the data were processed using Imaris 7.2.2 (Bitplane AG, Zurich,
256 Switzerland).

257

258 *Statistical analysis*

259 Means and standard deviations were calculated using Microsoft Excel 2007. Statistical
260 analyses were performed through Statistica[®]. To compare the antimicrobial effects of the
261 different treatments with the control (water), the Dunnett test was applied. Graphs were
262 performed with Microsoft Excel 2007.

263

264 **Results and discussion**

265 *Antimicrobial properties of wine and dealcoholized red wine on the biofilm*

266 The effects of a red wine and the same wine without ethanol on a biofilm model comprised of
267 five representative species commonly encountered in supragingival plaque, including Gram-
268 positive (*A. oris*, *S. mutans*, *S. oralis*) as well as Gram-negative (*F. nucleatum*, *V. dispar*)
269 bacteria (Guggenheim *et al.*, 2001), were investigated. Among these bacteria were the so-
270 called early colonizers, *A. oris*, *S. oralis* and *V. dispar*, and late colonizers, *S. mutans* and *F.*
271 *nucleatum*, the latter also designated as a bridging organism due to its capability to co-
272 aggregate with a wide range of early and late colonizers (Kolenbrander *et al.*, 2006). When
273 discs were dipped into both red wine and dealcoholized red wine, some decrease in cell
274 viability of the whole biofilm was visually estimated (Figure 2C) in comparison to the control
275 (Figure 2A). CFU values for the five bacteria comprising the biofilm indicated an important
276 reduction in *F. nucleatum* and *S. oralis* population when applying red wine and dealcoholized
277 red wine to the biofilm, in comparison to the negative control (distilled water) (Table 2). The
278 Dunnett test confirmed significant differences in the population of these two strains after the
279 treatment with wine and dealcoholized wine. Generally, wines contain between 10–12% of
280 ethanol, which have antimicrobial properties. To understand the action mechanism of red
281 wines in more depth, the effects of ethanol of the bacteria biofilm were investigated. The
282 treatment with 12% ethanol resulted in a significant decrease in the population of *F. nucleatum*

283 (Table 2). However, since treatments of the biofilm with both wine and dealcoholized wine
284 inhibited *F. nucleatum* growth, it was likely that other wine components – apart from ethanol –
285 had antimicrobial properties against this bacteria species. As expected, all the strains were
286 eradicated after the treatment with the positive control (0.2% chlorexidine-gluconate solution)
287 (Table 2).

288 In an intervention study with 75 volunteers, Signoretto *et al.* (2010) analyzed the microbial
289 population of supragingival and subgingival plaque using PCR-DGGE and found that *F.*
290 *nucleatum* was less frequentl in wine drinkers compared with water drinkers. Other authors
291 such as Daglia *et al.* (2007) have also shown antimicrobial properties of dealcoholized wine
292 against oral streptococci. Both studies were consistent with our results in that wine selectively
293 inhibited the growth of *F. nucleatum* and *S. oralis* in the presence of other species such as *S.*
294 *mutans*, *A. oris* and *V. dispar* in an oral biofilm model.

295 Given the antimicrobial effects of wine observed in the first experiment, the next step was to
296 study the influence of some wine-specific components such as polyphenols, including flavan-
297 3-ols, peptides or yeast polysaccharides. For that purpose, a red wine extract solution spiked
298 with different extracts rich in those specific components of wine (grape seed extract rich in
299 flavan-3-ols, and two inactive dry yeasts rich in peptides and mannoproteins, respectively)
300 were used. Table 3 reports the CFU values of the five bacteria species of the tested biofilm
301 after treatments with wine extract and wine extract solution spiked with different extracts
302 (grape seed extract, IDY1 and IDY2). Dunnett’s test showed significant differences in *F.*
303 *nucleatum*, *S. oralis* and *A. oris* with the application of the wine extract spiked with the grape
304 seed extract rich in flavan-3-ols (Monagas *et al.*, 2003). However, wine extract solutions
305 spiked with IDY1 and IDY2 did not show any effect in the populations of the five-strain
306 biofilm. Notably, a great decrease in the viability of the cell was visually appreciated in the
307 biofilm recovered from the discs that were dipped in the grape seed extract solution (Figure
308 2D). Cueva *et al.* (2012) reported significant inhibition in the growth of some oral streptococci,
309 such as *Streptococcus mutans* and *Streptococcus sobrinus*, when incubating planktonic cultures
310 with flavan-3-ols precursors, (+)-catechin and (-)-epicatechin, in which grape seed extract is
311 particularly rich. Moreover, they showed that extracts from grape seed, especially Vitaflavan®
312 and its oligomeric fraction, exerted higher antimicrobial activity against various oral pathogens
313 than the rest of the extracts tested (red wine extract and grape pomace extract). Similarly,

314 Rotava *et al.* (2009) and Baydar *et al.* (2006) reported antimicrobial effects of grape seed
315 extracts against pathogenic bacteria such as *S. aureus* and *E. coli*. It has been suggested that the
316 high concentration of flavonoids and their derivatives in grape seeds could be responsible of
317 the antimicrobial activity of grape seed extracts (Anastasiadi *et al.*, 2009). These observations
318 raise the question of how the hydroxyl groups (structure) of flavonoids affect oral bacterial
319 biofilm.

320 The search for new antimicrobial agents to control the formation of dental plaque requires
321 appropriate screening models that include orally relevant organisms. The model used in this
322 study is not only useful for investigating ecological shifts in plaque composition in response to
323 plaque composition but also for testing the efficacy of antimicrobial agents under conditions of
324 repeated short-term exposure (Guggenheim *et al.*, 2004).

325

326 *Change in wine phenolic metabolism*

327 Because wine and their polyphenols diminished bacteria population in the oral biofilm, a new
328 assay was performed in order to gain a deeper understanding about microbial metabolism of
329 polyphenols in the tested extracts.

330 Firstly, the wine extract solution was added to the growth media and the progress of the
331 phenolic metabolism by the five-species biofilm was studied by monitoring changes in the
332 main phenolic compounds present in the wine extract (Table 1), this is to say, flavan-3-ols
333 monomers ((+)-catechin, (-)-epicatechin and (-)-epicatechin 3-O-gallate, dimeric procyanidins
334 (B1, B2, B3, B4, B5, B7, B2-3-O-gallate and B2-3-O-gallate), trimeric procyanidins (C1 and
335 other trimers) and flavonols (quercetin, myricetin, kaempferol, quercetin-3-O glucoside and
336 quercetin-3-O galactoside)). As a brief example, Figure 3 shows the differences in the
337 degradation by the five-strain biofilm of three of the analyzed precursors, (+)-catechin,
338 procyanidin B2 and quercetin, when growing in media enriched with the wine extract solution
339 (1.6 g/L). The UPLC-MS analysis of these three compounds showed high degradation rates,
340 almost completely during the first 2 h of incubation, in the flavan-3-ol precursors, (+)-Catechin
341 and Procyanidin B2, probably because of their low concentration in the media which permitted
342 the bacteria of the biofilm using them as a carbon source. However, no degradation of the
343 precursor quercetin was observed during the incubation period.

344 To gain further knowledge about the metabolism of grape polyphenols, specifically in flavan-
345 3-ols metabolism, the growing media was enriched by adding, to the red wine extract solution,
346 a concentration of 10 g/L of grape seed extract, which is especially rich in flavan-3-ol
347 precursors. Despite the greater concentration of flavan-3-ol precursors, no degradation of the
348 flavan-3-ol precursors was observed, which could be associated with the high concentration of
349 the studied compounds that, in fact, had an antimicrobial effect on three out of the five bacteria
350 comprising the biofilm, as demonstrated in the antimicrobial assay (previous section). No
351 degradation of precursor quercetin was observed during the incubation period.

352 The emergence of antibiotic resistance by some oral bacteria biofilm species presents a
353 worldwide problem, and thus novel strategies are required. The use of natural antimicrobials
354 may contribute to controlling the disordered growth of oral microbiota, thus overcoming
355 problems caused by species resistant to conventional antimicrobials (Nascimento *et al.*, 2000).
356 To our knowledge, this is the first report on the antimicrobial properties of wine in an oral
357 biofilm model. Our results show that red wines, consumed in moderation, inhibit the growth of
358 some pathogenic species in an oral biofilm model. These findings contribute to existing
359 knowledge about the beneficial effects of red wines (one of the most important products of
360 agriculture and food industries) on human health. Moreover, the promising results concerning
361 grape seed extract, which showed the highest antimicrobial activity, open promising ways
362 towards a natural ingredient in the formulation of oral care products specifically indicated for
363 the prevention of caries, due to its antimicrobial properties.

364

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372

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Table 1. Main phenolic compounds in wine and extracts.

	Wine (mg/L) (Muñoz-González et al., 2013)	Wine extract (mg/g) (Sánchez-Patán et al., 2012)	Grape seed extract (mg/g) (Sánchez-Patán et al., 2011)
Benzoic acids			
Gallic acid	27.3±0.2	1.06 ± 0.05	9.11 ± 0.01
Protocatechuic acid	3.88±0.01	<i>n.a.</i>	<i>n.a.</i>
3-O-Methylgallic acid	1.06±0.06	<i>n.a.</i>	<i>n.a.</i>
4-Hydroxybenzoic acid	0.570±0.008	<i>n.a.</i>	<i>n.a.</i>
Vanillic acid	1.85±0.03	<i>n.a.</i>	<i>n.a.</i>
Syringic acid	2.30±0.13	<i>n.a.</i>	<i>n.a.</i>
Benzoic acid	1.14±0.06	<i>n.a.</i>	<i>n.a.</i>
Salicylic acid	0.215±0.001	<i>n.a.</i>	<i>n.a.</i>
Phenols			
Phloroglucinol	0.326±0.030	<i>n.a.</i>	<i>n.a.</i>
Tyrosol	31.4±1.4	18.9 ± 1.3	<i>n.a.</i>
Dihydroxyphenylpropan-2-ol	0.303±0.045	<i>n.a.</i>	<i>n.a.</i>
Cinnamic acids			
Caffeic acid	6.97±0.26	<i>n.a.</i>	<i>n.a.</i>
p-Coumaric acid	1.39±0.02	<i>n.a.</i>	<i>n.a.</i>
Ferulic acid	0.217±0.018	<i>n.a.</i>	<i>n.a.</i>
Coutaric acid	8.64±0.01	2.00 ± 0.12	<i>n.a.</i>
Caftaric acid	4.98±0.33	0.192 ± 0.071	<i>n.a.</i>
Stilbenes			
Resveratrol	7.12±0.29	0.427 ± 0.020	<i>n.a.</i>
Resveratrol-3-O-glucoside	<i>n.a.</i>	9.17 ± 0.17	<i>n.a.</i>
Flavan-3-ols and others			
(+)-Catechin	51.6±1.7	9.90 ± 0.32	74.6 ± 0.09
(-)-Epicatechin	34.9±2.9	6.87 ± 0.15	67.7 ± 0.75
(-)-Epicatechin-3-O-gallate	<i>n.a.</i>	0.226 ± 0.018	26.2 ± 0.41
Procyanidin B1	79.1±0.9	11.1 ± 0.1	61.0 ± 1.42
Procyanidin B2	44.7±0.6	4.69 ± 0.10	45.1 ± 0.95
B2-3-O-gallate	<i>n.a.</i>	0.0271 ± 0.0106	1.80 ± 0.06
B2-3'-O-gallate	<i>n.a.</i>	0.0258 ± 0.0028	1.61 ± 0.01
Procyanidin B3	16.0±1.0	1.23 ± 0.02	20.4 ± 0.33
Procyanidin B4	12.9±0.3	0.827 ± 0.018	15.0 ± 0.13
Procyanidin B5	2.67±0.01	<i>n.a.</i>	<i>n.a.</i>
Procyanidin B7	5.75±0.15	<i>n.a.</i>	<i>n.a.</i>
Procyanidin C1	14.0±0.4	1.07 ± 0.04	7.07 ± 0.08
Other trimers	7.96±1.05	1.24 ± 0.09	6.81 ± 0.06 (t2)
Flavonols			
Quercetin	1.92±0.01	22.4 ± 0.6	<i>n.a.</i>
Myricetin	0.697±0.028	2.55 ± 0.07	<i>n.a.</i>
Kaempferol	<i>n.d.</i>	0.0366 ± 0.0055	<i>n.a.</i>
Quercetin-3-O-glucoside	<i>n.a.</i>	0.137 ± 0.023	<i>n.a.</i>
Quercetin-3-O-galactoside	<i>n.a.</i>	0.107 ± 0.006	<i>n.a.</i>
Anthocyanins			
Delphinidin-3-O-glucoside	2.58±0.11	0.568 ± 0.012	<i>n.a.</i>
Cyanidin-3-O-glucoside	0.761±0.041	0.265 ± 0.010	<i>n.a.</i>
Petunidin-3-O-glucoside	4.06±0.13	1.47 ± 0.03	<i>n.a.</i>
Peonidin-3-O-glucoside	18.9±2.0	1.78 ± 0.01	<i>n.a.</i>
Malvidin-3-O-glucoside	36.7±3.4	9.01 ± 0.50	<i>n.a.</i>

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*n.d._not detected
*n.a._not analyzed

470 **Table 2.** Mean (\pm SD) of Log₁₀CFU values for *S. mutans*, *S. oralis*, *A.oris*, *F. nucleatum*, *V.*
 471 *dispar* after treatments with water, ethanol 12% in water, wine, dealcoholized wine and 0.2%
 472 clorhexidine-gluconate.
 473

	<i>S.mutans</i>	<i>S. oralis</i>	<i>F.nucleatum</i>	<i>A.oris</i>	<i>V.dispar</i>
Water	8.09 \pm 0.09	8.42 \pm 0.17	5.90 \pm 0.89	8.40 \pm 0.32	7.36 \pm 0.37
Ethanol 12% in water	8.01 \pm 0.16	8.20 \pm 0.37	<1.30 \pm 0.00 ^a	8.75 \pm 0.64	7.92 \pm 0.12
Wine	7.89 \pm 0.07	5.77 \pm 0.63 ^a	<1.30 \pm 0.00 ^a	8.37 \pm 0.20	6.94 \pm 0.38
Dealcoholized wine	7.68 \pm 0.22	4.79 \pm 0.80 ^a	<1.30 \pm 0.00 ^a	8.24 \pm 0.07	7.12 \pm 0.88
Clorhexidine-gluconate 0.2%	<1.30 \pm 0.00 ^a				

474
 475 ^a Significant differences (Dunnett's test) in the population in comparison to the negative control
 476 (water).

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479 **Table 3.** Mean (\pm SD) of Log₁₀CFU values for *S. mutans*, *S. oralis*, *A. oris*, *F. nucleatum*, *V.*
 480 *dispar* after treatments with Provinols™, Provinols™ + Vitaflavan®, Provinols™ + IDY 1 and
 481 Provinols™ + IDY 2.
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	<i>S.mutans</i>	<i>S. oralis</i>	<i>F.nucleatum</i>	<i>A.oris</i>	<i>V.dispar</i>
Water + 2.5% DMSO	8.02 \pm 0.05	8.47 \pm 0.99	6.77 \pm 0.07	8.55 \pm 0.07	7.74 \pm 0.04
Provinols™	8.11 \pm 0.08	8.59 \pm 0.11	6.54 \pm 0.57	8.34 \pm 0.39	7.68 \pm 0.39
Provinols™ + Vitaflavan®	7.77 \pm 0.18	6.49 \pm 0.07 ^a	<1.30 \pm 0.00 ^a	<3.30 \pm 0.00 ^a	7.95 \pm 0.09
Provinol™ + IDY 1	8.18 \pm 0.03	8.60 \pm 0.01	7.13 \pm 0.13	8.89 \pm 0.02	8.15 \pm 0.15
Provinols™ + IDY 2	8.13 \pm 0.07	8.44 \pm 0.07	7.14 \pm 0.04	8.68 \pm 0.03	8.11 \pm 0.11

483
 484 ^a Significant differences (Dunnett's test) in the population in comparison to the negative control
 485 (water + 2.5% DMSO).

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491 **FIGURE LEGENDS**

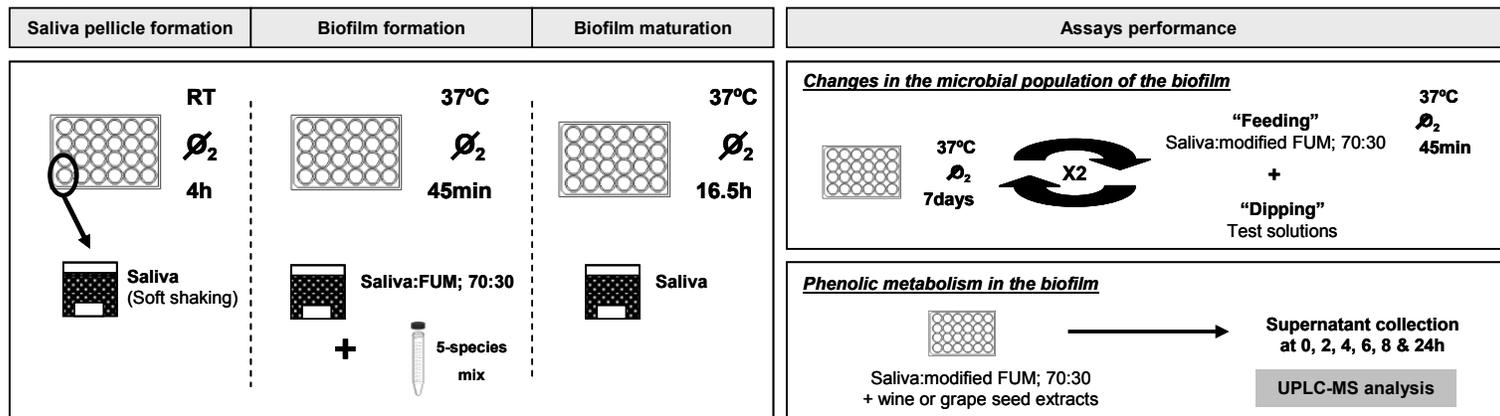
492 **Figure 1.** Biofilm formation/maturation and assays diagram

493 **Figure 2.** Confocal scanned segment of biofilm stained with LIVE/DEAD kit after exposure to
494 (A) negative control (water), (B) wine extract (Provinols™, 1.6 g/L), (C) red wine, and (D)
495 grape seed extract (Vitaflavan®, 2.5 g/L) in wine extract solution (1.6 mg/mL).

496 **Figure 3.** Metabolism of precursors (+)-Catechin, Quercetin and Procyanidine B2 after
497 0,2,4,6 8 and 24 hours of incubation in FUM media enriched with (A) Provinols™ and (B)
498 Vitaflavan® 1% in Provinols™ solution.
499

500 **FIGURES**

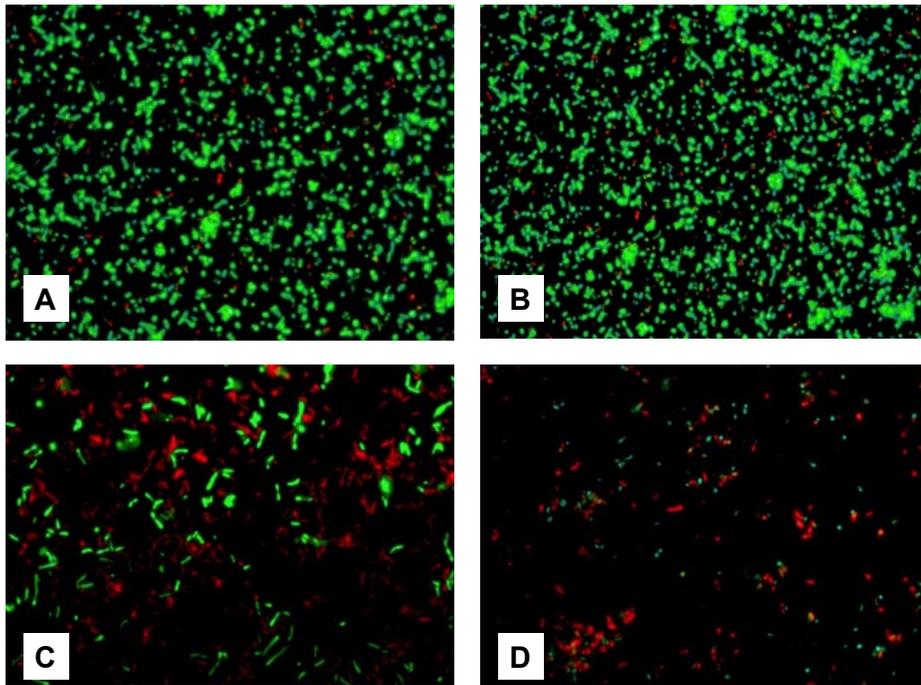
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503 **Figure 1.** Biofilm formation/maturation and assays diagram.

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506 **Figure 2.** Confocal scanned segment of biofilm stained with LIVE/DEAD kit after exposure to
507 (A) negative control (water), (B) wine extract (Provinols™, 1.6 g/L), (C) red wine, and (D)
508 grape seed extract (Vitaflavan®, 2.5 g/L) in wine extract solution (1.6 mg/mL).

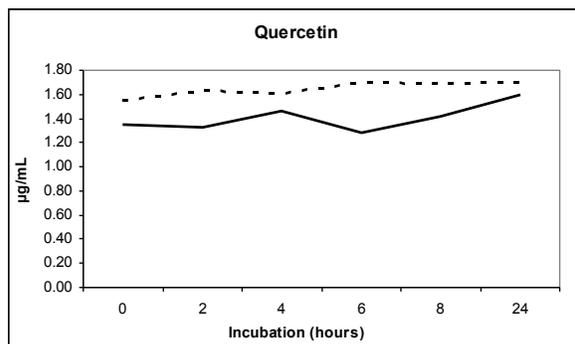
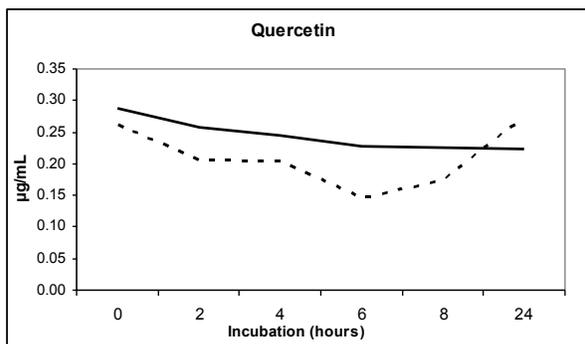
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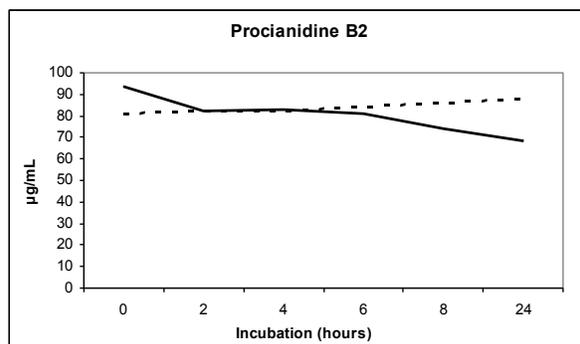
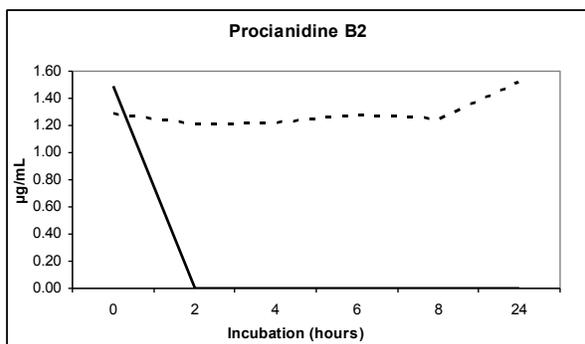
(A) Provinols™

(B) Vitaflavan® in Provinols™ solution



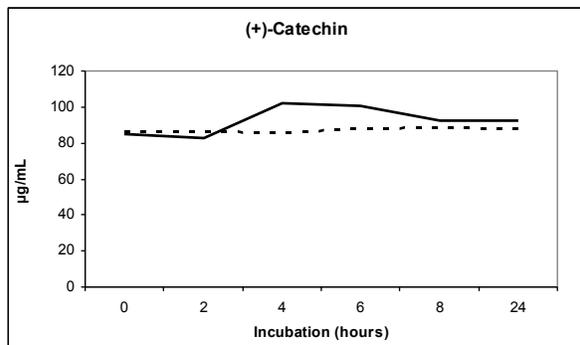
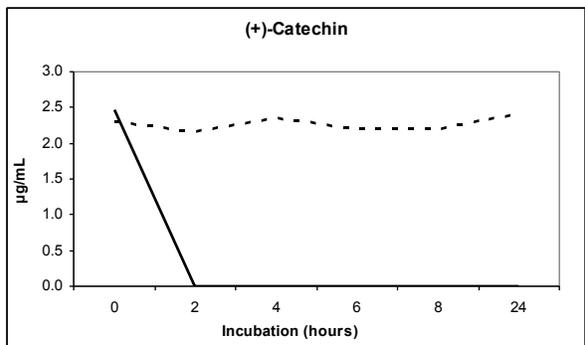
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518 **Figure 3.** Metabolism of precursors (+)-Catechin, Quercetin and Procyanidine B2 after
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520 Vitaflavan® 1% in Provinols™ solution.

521